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(71) Applicant (*for all designated States except US*): AMRAD OPERATIONS PTY. LTD. [AU/AU]; 576 Swan Street, Richmond, VIC 3121 (AU).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): SHORTMAN, Kenneth, Douglas [AU/AU]; The Walter and Eliza Hall Institute of Medical Research, PO Royal Melbourne Hospital, VIC 3050 (AU). SAUNDERS, Dolores, Joyous [CA/CA]; 39 Miro Court, Fredericton, New Brunswick E3B 5W2 (CA). LUCAS, Karen [AU/AU]; The Walter and Eliza Hall Institute of Medical Research, PO Royal Melbourne Hospital, VIC 3050 (AU). WU, Li [CN/AU]; The Walter and Eliza Hall Institute of Medical Research, PO Royal Melbourne Hospital, VIC 3050 (AU).

(74) Agents: HUGHES, E., John, L. et al.; Davies Collison Cave, 1 Little Collins Street, Melbourne, VIC 3000 (AU).

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(57) Abstract

The present invention relates generally to a cell culture process and to cells produced therefrom. More particularly, the present invention provides a method of developing dendritic cells from cultured precursor cells. The dendritic cells of the present invention are useful *inter alia* as adjuvants, immune system modulating agents, anti-cancer agent, immunotherapeutic agents and tolerizing agents for transplantation. The cell culture process of the present invention may also be extrapolated to a method of stimulating development of dendritic cells *in vivo*.

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A PROCESS FOR CULTURING CELLS

The present invention relates generally to a cell culture process and to cells produced therefrom. More particularly, the present invention provides a method of developing dendritic cells from cultured precursor cells. The dendritic cells of the present invention are useful *inter alia* as adjuvants, immune system modulating agents, anti-cancer agents, immunotherapeutic agents and tolerizing agents for transplantation. The cell culture process of the present invention may also be extrapolated to a method of stimulating development of dendritic cells *in vivo*.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Dendritic cells are sparsely but widely distributed migratory cells of bone marrow origin (Steinman, 1991). Their function has been considered to involve the collection of antigen in various tissue sites, transport of the processed antigen to the T cell areas of the lymphoid tissue, presentation of the antigenic peptides and stimulation of the immune response of T cells (Knight & Stagg, 1993).

Dendritic cells are generally considered to be of myeloid origin. Support for this view is based on studies of the outgrowth of dendritic cells, *in vitro*, under the influence of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Reid *et al.*, 1990; Inaba *et al.*, 1992; Caux *et al.*, 1992). Said dendritic cells appear to derive from a progenitor capable, also, of differentiating to granulocytes and macrophages (Inaba *et al.*, 1993). The myeloid nature of the dendritic cell is further supported by the direct

development of dendritic cells from blood monocytes (Romani *et al.*, 1994; Kabel *et al.*, 1989; Rossi *et al.*, 1992).

The dendritic cells of the thymus (Fairchild & Austyn, 1990) are believed to be the mediators of thymic negative selection, the process of elimination of potentially self reactive cells before mature T cells emmigrate to the periphery. Thymic dendritic cells are a relatively sparse population (0.1% of thymocytes) with a rapid turnover (7-day life span) (Kampinga *et ai.*, 1990; Wu *et al.*, 1995). Until the present invention, they were considered to arrive in the thymus preformed from the blood stream.

Adoptive transfer of highly purified precursor cells isolated from murine thymus has demonstrated that some thymic dendritic cells arise *in situ* from an intra-thymic precursor and are related to the lymphoid lineage. Said intra-thymic precursor appears identical to the earliest T cell precursor population in the adult thymus, the "low CD4" precursor. The low CD4 precursor population differs from multi-potent stem cells in its developmental potential in that although it retains the capacity to form B cells and NK cells, as well as T cells, it has lost the capacity to form myeloid or erythroid cells (Wu *et al.*, 1991; Ardavin *et al.*, 1993; Wu *et al.*, 1995). It is therefore a "lymphoid restricted" precursor.

By virtue of their highly developed antigen presenting capacity, the use of dendritic cells as cellular vectors for anti-tumour and infectious disease vaccines or as inducers of transplantation tolerance would be most beneficial. However, until the advent of the present invention, thymic precursors and precursors related to the lymphoid lineage could not be cultured *in vitro* to differentiate to dendritic cells.

In working leading up to the present invention the inventors have developed an *in vitro* process for developing dendritic cells from thymic precursor cells and more particularly from low CD4 precursor cells and pro-T cells.

Accordingly, one aspect of the present invention contemplates a method of developing dendritic cells from precursor cells, said method comprising culturing said precursor cells in the presence of an effective amount of a mixture of at least three cytokines or functional derivatives thereof wherein at least two cytokines are TNFα and IL-1.

More particularly the present invention contemplates a method of developing dendritic cells from precursor cells, said method comprising culturing said precursor cells in the presence of an effective amount of a mixture of at least three cytokines or functional derivatives thereof wherein at least three cytokines are TNFα, IL-1 and IL-3.

Reference hereinafter to a specific cytokine should be read as including reference to all forms of said cytokine and functional derivatives thereof. For example, reference to IL-1 should be understood to include reference to IL-1β and IL-1α and functional derivatives thereof.

Preferably, IL-1 is IL-1β.

Reference hereinafter to "dendritic cells" should be read as including reference to cells exhibiting dendritic cell phenotype or functional activity and mutants or variants thereof. "Variants" include, but are not limited to, cells exhibiting some but not all of the phenotypic features or functional activities of dendritic cells. Said phenotypic features may include expression of one or more of MHCII, CD11c, CD44, DEC-205, CD80 or CD86. Said functional activity includes, but is not limited to, the ability to stimulate the proliferation of allogeneic CD4 T cells in mixed leukocyte cultures. "Mutants" include, but are not limited to dendritic cells which are transgeneic wherein said transgeneic cells are engineered to express one or more genes encoding antigens, immune modulating agents, receptors or cytokines.

The term "precursor cells" should be understood to refer to cells which are not fully differentiated and which are derived from haematolymphoid tissue. Examples include, but are not limited to, precursor cells derived from bone marrow, spleen, lymph node,

thymus or blood. Said precursor cells are exemplified by but not limited to multi-potential stem cells, myeloid precursor cells or lymphoid precursor cells such as Lineage⁻ bone marrow derived precursors, $CD4^{lo}$ thymocytes, neonatal murine $CD4^-$ $CD8^-$ thymocytes and human $CD34^+$ $CD4^-$ $CD8^-$ thymocytes. Some or all of the precursor cells may also be transgenic in that said cells may be engineered to express one or more genes such as genes encoding antigens, immune modulating agents, receptors or cytokines. Preferably said precursor cells are lymphoid precursor cells.

Reference to the use of a "mixture" of cytokines according to the method of the present invention should be understood to encompass both the administration of a single composition comprising said cytokines or the sequential or simultaneous administration of one or more of said cytokines.

According to this preferred embodiment, the present invention provides a method of developing dendritic cells from lymphoid precursor cells, said method comprising culturing said precursor cells in the presence of a mixture of at least three cytokines or functional derivatives thereof wherein at least two cytokines are TNFα and IL-1.

More particularly the present invention provides a method of developing dendritic cells from lymphoid precursor cells said method comprising culturing said precursor cells in the presence of a mixture of at least three cytokines or functional derivatives thereof wherein at least three cytokines are TNFα, IL-1 and IL-3.

Even more particularly said IL-1 is IL-1β.

Preferably said lymphoid precursor cells are T cell precursor cells and even more preferably low CD4 precursor cells or pro-T cells derived from adult mouse thymus. Said low CD4 precursor cell is defined on the basis of the phenotypic profile $CD4^{lo}$, CD8-, CD3-, CD25+, CD45+c-kit+ while the pro-T cell is more differentiated than the low CD4 cell and therefore a downstream T cell precursor population defined on the basis

of phenotypic profile CD4-, CD8-, CD3-, CD25+, CD45+c-kit+.

Accordingly in a most preferred embodiment the present invention contemplates a method of developing dendritic cells from low CD4 precursor cells, said method comprising culturing said precursor cells in the presence of a mixture of least three cytokines or functional derivatives thereof wherein at least two cytokines are TNFα and IL-1.

More particularly in a most preferred embodiment the present invention contemplates a method of developing dendritic cells from low CD4 precursor cells, said method comprising culturing said precursor cells in the presence of a mixture of at least three cytokines or functional derivatives thereof which at least three cytokines are TNFα, IL-1 and IL-3.

Most particularly said IL-1 is IL-1β

In yet another most preferred embodiment the present invention contemplates a method of developing dendritic cells from pro-T cells said method comprising culturing said pro-T cells in the presence of a mixture of least three cytokines or functional derivatives thereof wherein at least two cytokines are TNFα and IL-1.

More particularly in another most preferred embodiment the present invention contemplates a method of developing dendritic cells from pro-T cells, said method comprising culturing said pro-T cells in the presence of a mixture of least three cytokines or functional derivatives thereof wherein at least three cytokines are TNFα, IL-1 and IL-3.

Most particularly said IL-1 is IL-1β

The present invention extends to any mixture of cytokines which is capable of facilitating development of dendritic cells from precursor cells, in particular from low

CD4 precursor cells and pro-T cells, and which contain at least TNFα, and IL-1. Preferably, the mixture comprises TNFα, IL-1 and IL-3 and even more preferably TNFα, IL-1β and IL-3. Most preferably the mixture comprises TNFα, IL-1β, IL-3 and an effective number of cytokines selected from IL-7, SCF, Flt3L and CD40 ligand (CD40L) or functional derivatives thereof.

"Functional derivatives" include fragments, parts, portions, chemical equivalents, mutants, homologs and analogs from natural synthetic or recombinant sources including fusion proteins. Chemical equivalents of said cytokines can act as functional analogs of said cytokines. Chemical equivalents may not necessarily be derived from said cytokines but may share certain conformational similarities. Alternatively, chemical equivalents may be specifically designed to mimic certain physiochemical properties of said cytokines. Chemical equivalents may be chemically synthesised or may be detected following, for example, natural product screenings.

Homologs of said cytokines contemplated herein include, but are not limited to, proteins derived from different species.

Derivatives may be derived from insertion, deletion or substitution of amino acids. Amino acid insertional derivatives include amino and/or carboxylic terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in said cytokines although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more amino acids from the sequence. Substitional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. Additions to amino acid sequences include fusions with other peptides or polypeptides. It is possible, for example, that the subject preferred cytokines may be substituted by other cytokines or hybrid cytokines antibodies directed to said cytokine receptor molecules, such as the CD40 antibody FGK45.5 or functional derivatives thereof. A hybrid cytokine may comprise a combination of cytokines.

Although not intending to limit the invention to any one method, cultures can be established from isolated low CD4 murine precursor cells using cell culture medium supplemented with a cytokine mixture said cytokines being TNFα, IL-1β, IL-3, IL-7, SCF, Flt3L, and CD40L. Said cultures exhibit initial cell death of approximately 25 to 30% of the total cell number. After one day of culture, cell division and expansion commences at which stage cells have already begun to display cytoplasmic extensions and a general dendritic cell morphology. Cell expansion is maximal at 4 days of culture, representing 4 to 5 times the original input, or around 7 times the initial surviving cell number. At this time the cells are in the form of tight clusters. Greater than 95% of the cells at this time have dendritic morphology. The number of viable cells declines after day 4 but a further five fold expansion can be achieved by splitting the cultures at day 3 to 4 and re-culturing in fresh medium with fresh cytokines.

Culturing murine pro-T cells under identical conditions similarly results in an extensive loss of cell viability over the first day amounting to approximately 70% of the initial cell input. After the initial cell drop, sub-division and expansion commence and cells of dendritic morphology appear in the cultures. Cell expansion peaks at day 4 where cells are observed to form tight clusters and greater than 96% of said cells exhibit dendritic morphology. Dendritic cell expansion and development proceeds from the viable cells surviving following the first day of culture.

In a particular preferred embodiment said cytokine mixture comprises TNFα, IL-1β, IL-3, IL-7, SCF and Flt3L or functional derivatives thereof.

Another preferred cytokine mixture comprises TNFα, IL-1β, IL-3, IL-7 and SCF or functional derivatives thereof.

Still another preferred cytokine mixture comprises TNFα, IL-1β, IL-3, IL-7, SCF, Flt3L and CD40L or functional derivatives thereof.

Yet another most preferred mixture comprises TNFα, IL-1β, IL-3, IL-7, SCF, Flt3L and anti-CD40 antibody or functional derivative thereof.

A most preferred mixture comprises TNFα, IL-1β, IL-3, IL-7, SCF, Flt3L and FGK45.5 or functional derivatives thereof.

The process of the present invention may be homologous or heterologous with respect to the animal or avian species from which the precursor cells and cytokines are derived. A homologous process means that the species from which the precursor cells are derived is the same as the species from which the cytokines are derived. For example, murine cytokines used to induce dendritic cell development from murine precursor cells. A heterologous process is one where at least one cytokine in the mixture of cytokines employed is from a species different to the species from which the precursor cells are derived. For example, one or more human cytokines used to induce dendritic cell formation from murine precursor cells or vice versa.

The present invention further contemplates a mixture for use in developing dendritic cells from precursor cells said mixture comprising at least three cytokines or functional derivatives thereof wherein at least two cytokines are TNFα and IL-1.

More particularly the present invention contemplates a mixture for use in developing dendritic cells from precursor cells said mixture comprising at least three cytokines or functional derivatives thereof wherein at least three cytokines are TNFα, IL-1 and IL-3.

Most particularly said IL-1 is IL-1β.

Preferably said mixture comprises the cytokine mixtures as hereinbefore described.

Even more preferably said precursor cells are low CD4 precursor cells or pro-T cells.

The present invention further extends to using the mixtures of cytokines to induce dendritic cell development *in vivo*. Such a method may be particularly important for immune compromised individuals, subjects undergoing chemotherapy or radiation therapy, subjects undergoing transplantation procedures or subjects having cancer or a disease condition.

Accordingly, another aspect of the present invention contemplates a method of treating a subject said method comprising administering to said subject an effective amount of a mixture of at least three cytokines or functional derivatives thereof for a time and under conditions wherein said cytokines induce dendritic cell development from precursor cells.

Preferably at least two cytokines are TNFα and IL-1.

Even more preferably at least three cytokines are TNFα, IL-1 and IL-3.

Most preferably said IL-1 is IL-1β.

In yet another aspect the present invention contemplates the use of a mixture of at least three cytokines in the manufacture of a medicament for the induction of dendritic cell development from precursor cells in a subject wherein at least two cytokines are TNFα and IL-1.

More particularly the present invention contemplates the use of a mixture of at least three cytokines in the manufacture of a medicament for the induction of dendritic cell development from precursor cells in a subject wherein at least three cytokines are TNFα, IL-1 and IL-3.

Most particularly said IL-1 is IL-1β.

Another aspect of the present invention contemplates a pharmaceutical composition comprising a mixture of at least three cytokines or functional derivatives thereof wherein said cytokines induce dendritic cell development from precursor cells together with one or more pharmaceutically acceptable carriers and/or diluents.

Preferably at least two cytokines are TNFα and IL-1.

Even more preferably at least three cytokines are TNFα, IL-1 and IL-3.

Most preferably said IL-1 is IL-1β.

Said cytokines or functional derivatives thereof are referred to as the active ingredients.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) and sterile powders for the extemporaneous preparation of sterile injectable solutions. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by appropriate action to reduce microbial

contamination, for example, the formulation may be filtered. Alternatively, a formulation may be prepared using sterilised components. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 μg and 2000 mg of active compound. Alternative dosages include from about 1 μg to about 100 mg and from about 10 μg to about 500 mg.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the

dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations. The effective amount is that amount of individual or combined cytokines effective to induce dendritic cell development from haemopoietic cells. Proposed effective amounts include but are not limited to from about 0.01 μg/kg body weight to about 1000 mg/kg body weight, or from about 700 mg/kg body weight or from 1 μg/kg body weight to about 500 mg/kg body weight. These amounts may be combined cytokine amounts or the amounts of individual cytokines used.

The pharmaceutical composition may also comprise genetic molecules such as a vector capable of transfecting target cells where the vector carries a nucleic acid molecule capable of modulating the expression of a nucleotide sequence encoding one or more cytokines or functional equivalent thereof. The vector may, for example, be a viral vector.

The present invention is exemplified herein with respect to murine precursor cells and murine dendritic cells. However, this is done with the understanding that the present invention extends to all animals including humans, livestock animals (e.g. sheep, cows, horses, donkeys), laboratory test animals (e.g. rats, guinea pigs, rabbits, hamsters), companion animals (e.g. dogs, cats), captive wild animals (e.g. emus, kangaroos, deer, foxes) and birds (e.g. chickens, ducks, bantams, pheasants, emus, ostriches).

In yet another aspect the present invention contemplates the use of dendritic cells in the manufacture of a medicament for the treatment of a mammal.

The methods and compositions of the present invention are useful for generating

dendritic cells for use in a range of therapeutic and diagnostic procedures. For example, the dendritic cells may be used as cellular vectors for anti-tumour and infectious disease vaccines or as inducers of transplantation tolerance. These strategies are based on the highly developed antigen presenting capacity of dendritic cells. The dendritic cells of the present invention may also act as adjuvants for enhancing an immune response to, for example, tumour cells or other antigens such as those derived from prokaryotes, eukaryotes, or viruses, including human immuno deficiency viruses (e.g. HIV-I), influenza viruses and hepatitis viruses (e.g. Hepatitis A, B and C). In this regard, the dendritic cells may also be loaded with such antigens to induce tolerance. The dendritic cells of the present invention may also be used as regulators of the immune response for example by down-regulating T cell activity or skewing the nature of a T cell response. Said down-regulation has applicability in the therapeutic or prophylactic treatment of disease conditions involving an unwanted immune response such as autoimmune conditions. Said skewing includes, for example, skewing a Th1 dominated immune response to a Th2 dominated immune response.

The dendritic cells produced by the method of the present invention may be fused with other cell types such as, for example, a tumour cell. Said fused dendritic cell is useful in a range of therapeutic and prophylactic procedures. For example, the processing and presentation of tumour antigens by a dendritic cell fused with a tumour cell has application as a therapeutic or prophylactic vaccine.

According to this aspect of the present invention said use of dendritic cells may be "syngeneic", "allogeneic" or "xenogeneic" with respect to the individual undergoing treatment and the individual from which said precursor cells are initially derived. A syngeneic process means that the individual from which the precursor cells are derived has the same MHC genotype as the individual receiving treatment. An allogeneic process means the precursor cells are derived from an individual which is MHC incompatible in relation to the individual receiving treatment. For example, said precursor cells are derived from a Balb/C mouse while the individual receiving

treatment is a CBA mouse. A xenogeneic process means that the individual from which the precursor cells are derived is of a different species to the individual receiving treatment. For example, a primate is receiving treatment utilising dendritic cells derived from murine precursor cells.

Another aspect of the present invention contemplates a method of treating a subject said method comprising administering to said subject an effective number of dendritic cells wherein said dendritic cells are produced by the method of the present invention.

A further aspect of the present invention contemplates dendritic cells produced by the method of the present invention.

Further features of the present invention are more fully described in the following Examples and Figures. It is to be understood, however, that this detailed description is included solely for the purposes of exemplifying the present invention. It should not be understood in any way as a restriction on the broad description of the invention as set out above.

The present invention is further described by the following non-limiting Figures and Examples.

In the Figures:

Figure 1 is a graphical representation showing the stimulation of CD4 T cell proliferation by the dendritic cell derived in culture from the thymic low CD4 precursors. The cultured dendritic cells were harvested on day 4 from cultures of thymic low CD4 precursors grown in the presence of the cytokines IL-1β, TNFα, IL-3, IL-7, SCF, Flt3L and the mAb FGK45.5 reactive with CD40. These were compared with normal thymic dendritic cells extracted directly from the thymus, and finally

purified by sorting based on CD11c expression. Purified CBA lymph node CD4 T cells (20,000) were cultured for 3 days with various levels of the C57BL/6 derived dendritic cells, then the cultures pulsed for 9 hr with ^{3}H-TdR. The cells were collected onto glass- fibre filters and proliferation was assessed by measuring incorporated radioactivity using gas-flow scintillation counting. Results are the means ± SEM of the pooled data from two experiments, each with 5 cultures per point. Similar results but with somewhat lower counts were obtained at day 2.5 and 3.5 of harvest. The background count with T cells alone was 17 ± 1 cpm, the stimulation index was over 300. The background count with 2000 fresh thymic dendritic cells alone was 77 ± 22 cpm and with 2000 cultured dendritic cells alone was 109 ± 14 cpm.

Figure 2 is a graphical representation of the expression of CD8α on the dendritic cells produced in spleen by transfer of various precursor populations. Enriched splenic dendritic cell preparations were stained for CD8α, class II MHC and Ly 5.2 expression; the histograms represent the level of CD8α on the dendritic cells, gated as high class II MHC cells with characteristic dendritic cell light scatter, and gated as Ly 5.2$^+$ cells of donor origin. The broken lines gives the background fluorescence omitting only anti-CD8α. The upper graph demonstrates the presence of both CD8α$^-$ and CD8α$^+$ dendritic cells in the spleens of normal mice. The second graph shows that the dendritic cell progeny of transferred bone marrow (BM) have a similar distribution of Cd8α$^-$ and CD8α$^+$ dendritic cell. The lower graphs demonstrate that the early thymic precursors generate only CD8α$^+$ dendritic cells in the spleen following intrvenous transfer. This illustrates the selective production of one type of dendritic cell from the thymic precursors.

EXAMPLE 1

Mice

The mice used for isolation of thymic low CD4 precursors, or for isolation of thymic dendritic cells, were usually 5-7 wk-old C57BL/6J Wehi females, bred under specific pathogen-free conditions at The Walter and Eliza Hall Institute animal facility. The GM-CSF null mice, produced at the Ludwig Institute, Melbourne (Stanley *et al.*, 1994), were originally on a C57BL/6 x 129 background but had been backcrossed for 5 generations onto C57BL/6J mice; 5-9 wk old males and females were used. The source of the $CD4^{+}$ lymph node T cells for mixed leukocyte reactions was 5-6 wk female CBA/J mice bred under specific pathogen-free conditions at The Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria, Australia.

Female C57BL/6J Wehi mice, 5-7 wks of age, bred under specific-pathogen-free conditions at The Walter and Eliza Hall Institute Animal Facility, were also used for the culture studies. In the cell transfer studies the precursor cells were isolated from C57BL/6 (Ly 5.2) mice and transferred into irradiated C57BL/6 Ly 5.1 Pep^{3b} mice as described fully elsewhere (Wu *et al.*, 1996; Wu *et al.*, 1991).

EXAMPLE 2

Isolation of Normal Dendritic Cells from the Thymus

The procedure was modified from that given in detail elsewhere (Vremac *et al.*, 1992; Suss & Shortman, 1996; Winkel *et al.*, 1994). Briefly, pooled thymuses from 10 mice were cut into fragments and the entire tissue digested for 25 min at 22°C with collagenase DNase. The digest was incubated a further 5 min with EDTA to break up dendritic T cell complexes. Light density cells were then isolated from the digest by centrifugation at 4°C in a 1.077g/cm^{3} density medium isoosmotic with mouse serum. The light density cells were then coated with a cocktail of mAb reactive with CD3, CD4, Thy 1, CD25, B cell antigen B220, erythrocyte antigen TER119, granulocyte

antigen Gr-1, macrophage antigen F480, FcRII and CD11b, then the coated cells removed using anti-immunoglobulin coated magnetic beads. Finally the dendritic cells in the enriched preparation were stained and stored as cells excluding propidium iodide, with the high forward and side scatter of dendritic cells, and expressing relatively high levels of CD11c (Suss & Shortman, 1996).

EXAMPLE 3

Isolation of precursor cells from the thymus

Low CD4 - 2 methods were utilised:

(a) An improved procedure was used, modified from original method (Wu *et al.*, 1991) and given in detail elsewhere (Ismaili *et al.*, 1996). Briefly, the lightest 30% of thymocytes were selected by a density cut procedure, then all adherent cells removed by culture for 1 hr in Petrie dishes. Cells bearing CD3, CD8, CD25, B220, class II MHC, Gr-1, Mac-1 and the erythrocyte antigen TER 119 were all removed by first coating with mAb, then removing coated cells with two rounds of anti-immunoglobulin coated magnetic beads. Finally, the 1% remaining thymocytes were stained with anti-Thy 1 (PE-conjugated 30.H12) and anti c-kit (FITC-conjugated ACK-2). The low CD4 precursors were sorted as cells low but positive for Thy 1 and moderate to strongly positive for c-kit. The preparation was >97% pure on reanalysis by these markers and appeared homogenous by 14 other markers tested in previous experiments (Wu *et al.*, 1991; Ardavin *et al.*, 1993; Wu *et al.*, 1995; Ismaili *et al.*, 1996; Shortman & Wu, 1996).

(b) The procedures have been described in full elsewhere (Wu *et al.*, 1991). The low CD4 precursors were isolated by first removing from thymus suspensions the 70% most dense cells, then adherent cells, and then by immunomagnetic bead depletion removing mature T cells, $CD4^{+}8^{+}$ thymocytes, most of the downstream precursors, and mature non-T lineage cells including dendritic

cells. Anti-CD4 was not employed in the depletion mAb cocktail. These precursors were then fluorescent labelled and finally positively sorted as cells which were low for Thy 1 and medium/high for c-kit.

Other Thymic precursors:

(c) The other precursors were isolated by first depleting thymus suspensions for cells bearing CD4, CD8 or CD3, as well as for mature non-T lineage cells including dendritic cells. The precursors were then fluorescent labelled and positively sorted as cells which were c-kit$^+$ CD25$^+$, or which were c-kit$^-$CD25$^+$ or which were c-kit$^-$CD25$^-$.

EXAMPLE 4

Culture Conditions

The culture medium was based on RPMI 1640, modified to be isoosmotic with mouse serum (308m.osmolar), with additional Hepes-buffering at pH7.2, and supplemented with 10% v/v FCS, 10^{-4}M 2-ME, sodium pyruvate and antibiotics. The required cytokines were then added to the medium and the precursor cells dispersed in the mix. Cultures were from 1-7 days at 37.5°C in a humidified 10% v/v CO_2-in-air incubator. For most studies the culture volume was 0.01ml, and culture of 1-3000 cells was in the wells of Terasaki trays (Nunclon, Denmark). When a larger cell yield was required for cell counts or for surface phenotype analysis, 20,000 precursors were cultured in 0.1 ml medium in flat-bottomed 96-well culture trays (Disposable Products Pty Ltd, South Australia).

EXAMPLE 5

Cytokines and Cytokine-related Antibodies

The following concentrations of recombinant cytokines tested were used for culture of the low CD4 precursors:

(a) IL-1β (human), 200U or 0.2ng/ml; tumor necrosis factor (TNF)α (murine), 1ng/ml; IL-3 (murine), 200U or 400 ng/ml; IL-4 (murine), 200U or 20 ng/ml; IL-7 (human), 200U or 10ng/ml; GM-CSF (murine), 200 "Immunex units" or 200ng/ml. GM-CSF was found by titration at the end of the experimental series to be 3200 "standard units" or the equivalent of 16 ng/ml of a standard Hall Institute preparation.

(b) The medium was modified RPMI-1640, isoosmotic with mouse serum, containing additional HEPES-buffering at pH 7.2 and supplemented with 10% fetal calf serum, 10^{-4}M 2-mercaptoethanol, sodium pyruvate and antibiotics. The full complement of seven cytokines was used, namely: TNFα 1ng/ml; IL-1β 0.2 ng/ml; IL-3 400 ng/ml; IL-7 100 ng/ml; SCF 10 ng/ml; Flt3 ligand 100 ng/ml; mAb against CD40, FGK45.5, 1μg/ml. Cultures were incubated at 37.5°C in a humidified 10% CO_2-in-air gas phase.

These cytokines were all provided by Immunex Corp., Seattle, WA, except for Flt3/Flk2 ligand (Flt3L), 100ng/ml, and antibody against GM-CSF, 2μg/ml, which were provided by Dr N Nicola, The Walter and Eliza Hall Institute of Medical Research Melbourne, Victoria, Australia, CD40 ligand (CD40L) and mAb against CD40, FGK45.5, 1μg/ml, which were provided by Dr A Rolink, Basel Institute for Immunology, Basel, Switzerland.

EXAMPLE 6

Cluster Counts, Cells Counts and Visualization of Dendritic Morphology

The incidence of dendritic cell clusters was counted directly on the Terasaki tray cultures, using inverted phase-contrast microscopy; a group of more than 20 cells was considered a cluster. To recover and count cells after culture one tenth volume of 0.1M EDTA, pH 7.2, was first added to the warm cultures, then the cultures were mixed by repeated passage through a pipette tip in order to break up the dendritic cell clusters into a single cell suspension; cell counts were then carried out in a hemocytometer, using phase

contrast microscopy. To assess dendritic morphology, a cell suspension was prepared from pooled cultures using EDTA to aid dissociation, as above. The cells were then washed by centrifugation through a layer of foetal calf serum, and resuspended in a small volume of culture medium. The suspension was placed in slide chambers, prepared by fastening square coverslips onto microscope slides by double-sided adhesive tape at two opposite edges. After filling the chambers the remaining edges were sealed with nail polish. The slides were then incubated at 37°C for 1-2 hr, then examined under phase-contrast microscopy. To monitor the fate of individual precursor cells, Teraskai tray cultures containing only a single precursor were selected after 2 hr of incubation of cultures set up using 1 cell per 0.01 ml medium, then the culture was inspected every 24 hr using inverted phase-contrast microscopy.

EXAMPLE 7

Immunofluorescent Staining and Flow Cytometry

The procedures used for staining the cultured dendritic cells were similar to those used previously for dendritic cells extracted from tissues (Vremac *et al.*, 1992). The dendritic cells were harvested from the cultures at day 4 as above pooled, then stained with directly-conjugated mAb in two of three fluorescent colors, together with propidium iodide in order to exclude dead cells. They were then analyzed using a FACStar Plus (Becton Dickinson, San Jose, CA). The mAb and the fluorochromes used were: CD4, Cy5-conjugated H129.19.6.8; CD8α, biotin-conjugated 53-6.7; CD8β, biotin conjugated 53-5.8; CD3, PE-conjugated KT3-1.1; class I MHC, biotin-conjugated M1/42; class II MHC, Texas Red-conjugated N22; CD11c, biotin-conjugated N418; DEC205, FITC-conjugated NLDC145; BP-1, biotin-conjugated 6C3; CD11b, FITC-conjugated M 1/170; F480 macrophage antigen, biotin-conjugated F4/80; B220, biotin-conjugated RA3-6B2; CD80 (B7/1), biotin-conjugated 16-10A1; CD86(B7/2), biotin-conjugated GL-1; CD40, FITC-conjugated FGK45.5; CD44, FITC-conjugated IM7.81; Gr-1, FITC-conjugated RB6-8C5; CD25, biotin-conjugated PC61. PE-conjugated steptavidin was used as the second stage for all biotin conjugates (Wu *et al.*, 1991; Wu *et al.*, 1995; Vremec *et al.*, 1992).

EXAMPLE 8

Mixed Leukocyte Cultures for Assessing CD4 T cell Stimulatory Capacity

The cultures were set up and T cell proliferation determined as described previously (Suss & Shortman, 1996). Briefly, 100-2000 dendritic cells of C57BL/6 origin, either harvested from the cultures or isolated from the thymus, were cultured with 20,000 purified CD4 T cells isolated from the lymph nodes of either CBA, C3H or C3H lpr mice. The culture medium was modified RPMI-1640, 0.1 ml being used in the wells of V-bottom 96-well culture trays. No exogenous cytokines were added. After 2-4 days at 37.5°C in a 10%v/v-CO_2-in-air incubator, the cultures were pulsed for 9 hr with ^{3}H-TdR. Cells in the cultures were harvested onto glass-fibre filters and incorporated radioactivity measured in a gas-flow scintillation counter.

EXAMPLE 9

Culture of Low CD4 Precursors with One or Two Cytokines

In the inventors' initial studies, the low CD4 precursors were isolated from adult mouse thymus by depletion then sorting, and then cultured at 50-1000 cells per well in Terasaki tray cultures with a range of recombinant cytokines. The cytokines were tested singly, or in combinations of two or sometimes three. In no case of cytokines used alone or in pairs was any growth detected. This included the cytokines normally used to produce dendritic cells in culture, namely GM-CSF, or GM-CSF in combination with TNFα and/or IL-4. It also included IL-2 and IL-6, not used in subsequent studies.

However, almost all the cytokines, even when used singly, gave some improvement in low CD4 precursor survival. After 36 hr of culture in medium alone, an average of only 10% of the precursors were viable. The cytokines which when used alone increased survival to above 30% where IL-3, IL-6, SCF, TNFα, IL-4 and GM-CSF; IL-7 gave the best survival, 55%. No combination of cytokines gave a survival above 45%. Some combinations of cytokines reduced survival to that of the medium alone, in particular TNFα with GM-CSF, TNFα with SCF,TNFα with IL-4, and IL-4 with IL-7. This

indicated that low CD4 precursors expressed receptors for many of these cytokines but that the interactions between them were complex.

EXAMPLE 10

Growth and Differentiation of Low CD4 Precursors in Response to Multiple Cytokines

In contrast to this lack of proliferation in response to combinations of up to two cytokines, some growth and differentiation of the low CD4 precursors was obtained on thymic epithelial cell lines (Saunders *et al*, 1995). A complex cocktail of seven cytokines, including some that might have been produced by thymic epithelial cells, namely: TNFα, IL-1β, IL-3, IL-4, IL-7, SCF and GM-CSF was tested. This cytokine cocktail was tested on the purified precursors cultured alone, without the thymic epithelial cell underlay. Purified thymic low CD4 precursors were cultured for 4 days in 0.01 ml medium in Terasaki tray cultures. The number of clusters (>20 cells) per well was counted under phase contrast microscopy. It produced a definite growth of precursors, doubling the input cell number by day 3. A requirement for multiple cytokines to induce growth in the low CD4 precursors has also been reported by Moore and Zlotnik (1995). However, the end-product cells appeared to be dendritic cells in the cultures of the present invention. Cells with cytoplasmic extensions and dendritic cell morphology appeared by day 1. From day 2 to day 4 of culture a high proportion of the cells formed large clusters of around 50 cells, resembling closely the dendritic cell clusters generated by culturing bone-marrow or blood precursors with GM-CSF and other cytokines (Reid *et al.*, 1990; Inaba *et al.*, 1992; Caux *et al.*, 1992; Scheicher *et al.*, 1992; Reid *et al.*, 1992). The majority of cells in the cultures had the morphological appearance of dendritic cells by day 4.

The clusters appeared to form as a result of aggregation, rather than representing true colonies derived from single precursors. Nevertheless, over the range of 100-5000 low CD4 precursor cell input, there was a linear dose-response relationship between cells cultured and clusters formed at day 4, with 5-8 clusters being formed per 1000 cells cultured. Accordingly, scoring the number of large clusters formed in the Terasaki well

cultures provided a rapid assay for proliferation and dendritic cell production. With relatively dense cultures (3000 precursors per well) a statistically reliable estimate could be made with around 5 cultures per point. Such a cluster count was used as the initial readout for screening the contribution of different cytokines.

EXAMPLE 11

The Effect of Omitting Cytokines on Dendritic Cell Cluster Development

To determine which of the cytokines in the complex mix were essential, the effect of leaving out one or two individual cytokines from the initial mix was systematically assessed, using the incidence of dendritic cell clusters at day 4 as a readout. Thymic low CD4 precursor cells were cultured at 3000 cells per well in Terasaki culture plates, and the number of clusters of dendritic cells counted at day 4. Several cytokines (GM-CSF, IL4, IL-7, SCF and IL-3) could be omitted individually without much effect on cluster formation . However, their absence generally did have an effect if omitted along with another cytokine; one exception was the omission of GM-CSF and IL-4 together, where no drop was evident. The two cytokines whose omission, either alone or in combination with other cytokines, had the greatest effect were IL-1β and TNFα. When omitted together, cluster formation dropped to 8% of that seen with the complete mix. It was also notable that if GM-CSF was omitted, cluster formation became very dependent on IL-7. In contrast, the omission of both GM-CSF and IL-3, which share a common receptor chain (Nicola, 1994) and might therefore have substituted for one another, caused only a small drop in cluster formation.

Accordingly IL-1β and TNFα were considered essential components of the mix, whereas GM-CSF and possibly IL-4 appeared dispensable. To check this further. GM-CSF was omitted from the mix and the effects of omitting one or two further cytokines (except L-1β and TNFα) was examined. The further omission of IL-4 had no effect on cluster formation. IL4 was omitted from subsequent cytokine cocktails. The omission of IL-7 in the absence of GM-CSF now had a marked effect on cluster formation, although interestingly this drop was less when other cytokines, in particular IL-4, were absent.

EXAMPLE 12

Effect of Antibody Against GM-CSF on Development of Dendritic Cell Clusters

The lack of any requirement for GM-CSF in this production of dendritic cells was surprising, in view of its requirement for dendritic cell outgrowth in other systems. It was possible the low CD4 precursors themselves, or some trace contaminant, produced sufficient endogenous GM-CSF. To test this possibility, a neutralizing antibody against GM-CSF was added to the cultures when they were initiated, at a level known to block GM-SF-dependent colony-formation in culture. The cultures were stimulated by the above "optimal" mix of five cytokines, lacking GM-CSF. However, there was no significant drop in the number of dendritic cell clusters formed, nor any reduction in the apparent size of the clusters, when the antibody was added (Table 1). In view of the possibility that IL-3 was substituting for GM-CSF, since they share a common receptor chain (Nicola, 1994), the test was repeated with both IL-3 and GM-CSF omitted from the cytokine mix. Again the anti-GM-CSF had no significant effect in these relatively high density cultures.

EXAMPLE 13

Cytokine Requirements in Low Cell Density Cultures

Finally, to verify the requirement for all five cytokines, some simpler combinations were tested, at a lower precursor cell input (250 precursors per culture) to reduce any effects of endogenous growth factor production. The precursors were cultured in 0.01 ml medium in Terasaki tray wells. Cell counts were performed after harvest in a hemocytometer with viability assessed by appearance under phase-contrast microscopy. All simpler cytokine combinations gave fewer dendritic cell clusters, or no clusters at all (Table 2), and the few clusters that were obtained appeared smaller. Two aspects of these low cell density cultures were notable. First, neither dendritic cell cluster formation nor cell expansion was evident in cultures with IL-1β alone or TNFα alone, or IL-1β plus TNFα, even though these cytokines were essential for the growth of dendritic cells in the cytokine mix. However, in the cultures with IL-1β alone, or in cultures with IL-1β plus TNFα, approximately 20% of the individual, non-clustered surviving cells acquired dendritic

morphology, suggesting IL-1β alone promoted some direct dendritic cell differentiation without cell division. In accordance with this, a few dendritic cell clusters were obtained when very high density cultures were incubated in IL- 1β plus TNFα. A second aspect of the sparse cultures was the degree of dependence on IL-3 for dendritic cell cluster formation. In contrast to the dense cultures where omission of IL-3 had a smaller and variable effect (Table 1), omission of IL-3 from the sparse cultures caused a much greater drop in both dendritic cell cluster formation and in cell proliferation. A low level of endogenous IL-3 production by the precursor cells themselves is one possible explanation for this difference.

Under these low cell density culture conditions with the five cytokine mix, a net increase of the cells in the cultures was obtained, with growth extending to 5 days and reaching 3-4 times the original cell input. Over 90% of the cells harvested at days 3 to 4 of culture had dendritic morphology. In contrast, cultures of cells in medium without cytokines showed continuous cell death, no cell division and no cells with dendritic morphology.

EXAMPLE 14

The Effect of CD40 Ligation and Flt3 Ligand Addition on Dendritic Cell Development

Soluble CD40L has been found to enhance dendritic cell survival and differentiation (Caux *et al.*, 1994). Although it was ineffective alone, the present inventors found that it enhanced the dendritic cell development stimulated by the above five cytokine mix. In the presence of soluble CD40L, the cultured cells more rapidly attained the extreme dendritic cell form with extended dendrites, the number of clusters and their size was increased, the cell yield increased and fewer cells were found outside the clusters. The impression was of enhanced differentiation with an earlier peak of dendritic cell production. Very similar results, but more reproducible in the extent of the effect, were obtained by adding the mAb FGK45.5, reactive with CD40. Cultures of 250 purified low CD4 precursors were set up in 0.01 ml medium in Terasaki tray culture wells. This mAb was used instead of soluble CD40L in subsequent experiments.

Flt3L injected into mice induces a striking increase in the levels of all types of dendritic cells in mouse lymphoid organs. Although Flt3L was without effect on the low CD4 precursors alone, when added together with the previous five cytokine mix it enhanced dendritic cell development in culture. The number of cells produced in the cultures increased 1.6-fold and peaked a day earlier, while the clusters increased a little in both number and size. The progeny cells again had dendritic cell morphology, although of a less extreme form than with CD40L.

The addition of both Flt3L and the mAb ligating CD40 to the cytokine mix of TNFα, IL-1 β, IL-3, IL-7 and SCF, appeared to produce the optimal yield and morphological form of dendritic cells from the cultured low CD4 precursors, although these two additional "cytokines" were ineffective if used alone. With this new seven "cytokine" mix the numbers of cells reached 4-5 fold the initial input by day 4 of culture, this was a minimal estimate, since under these conditions the clusters were difficult to completely dissociate even with EDTA. The number and size of clusters was also maximised, and peaked at day 4, with this mix. Over 95% of the individual cells harvested and recovered from such cultures had the morphological appearance of dendritic cells, all having multiple fine cytoplasmic extensions and many having more obvious "dendrites".

EXAMPLE 15

CD4 Precursors from GM-CSF Null Mice

The surprising lack of any requirement for GM-CSF in dendritic cell generation required a more critical assessment. It was possible that traces of endogenously derived GM-CSF persisted in the cultures despite the antibody blocking experiments of Table 1. It was also possible that GM-CSF was required for the generation of the precursors from multipotent stem cells, rather than at the later dendritic cell developmental steps reflected in our cultures. Accordingly, the development in culture of the thymic low CD4 precursors derived from GM-CSF "null" mice was assessed, with the GM-CSF gene deleted by homologous recombination (Stanley *et al.*, 1994). The yield of low CD4 precursors from the thymus of the GM-CSF "null" mice was similar to that obtained from the normal

C57BL/6 control mice, indicating that the generation of this precursor population was independent of GM-CSF. When cultured at low cell density with the final complement of seven "cytokines" (lacking GM-CSF), the thymic precursors from the GM-CSF null mice showed extensive proliferation and produced dendritic cell clusters, with over 90% of the product cells showing typical dendritic morphology (Table 3). However, both the total cell expansion and the number of dendritic cell clusters was only 70-75% that obtained by culturing the same number of low CD4 precursors from normal mice. Thus, although dendritic cell development occurred efficiently in the absence of GM-CSF, it remained possible that traces of endogenous GM-CSF could enhance this development. Alternatively, GM-CSF in the normal mice may have slightly "conditioned" the precursors for an enhanced response, or removed some irrelevant cells from the "low CD4 precursor" pool.

EXAMPLE 16

Effects of IL-3 and GM-CSF on Dendritic Cell Development in Low Cell Density Cultures

The results with the GM-CSF null mice suggested that GM-CSF might have some stimulatory effect in low density cultures, despite its lack of effect in the earlier high precursor cell input studies. Another possibility was that the requirement for GM-CSF was being largely met by the added IL-3, via interaction with a common receptor β-chain; since the requirement for IL-3 only became pronounced in low density cultures (Table 2), it was important to recheck this issue under these conditions. Accordingly, low CD4 precursors were cultured with IL-1β, TNFα, IL-7, SCF, Flt3L and anti-CD40 mAb, then the effects of adding IL-3 and/or GM-CSF were examined (Table 4).

Precursor cell expansion and dendritic cell development occurred in the absence of both IL-3 and GM-CSF, with the vast majority of cultured cells having dendritic morphology and aggregating into clusters. However, the yield of both dendritic cell and dendritic cell clusters was about half that seen in the presence of IL-3. Therefore, GM-CSF could partially substitute for IL-3 under these conditions. However, GM-CSF did not synergize

with IL-3, as some inhibition in cell expansion was noted when both were added together. Similar but slightly reduced effects were obtained when GM-CSF was added to the cultures at a 10-fold lower concentration.

EXAMPLE 17

The Surface Phenotype of the Cultured Dendritic Cell

Immunofluorescent staining and flow cytometry was used to analyze the surface antigens on the cells obtained from day 4 cultures of low CD4 precursors grown in the mix of TNFα, IL-lα, IL-3, IL-7 and SCF, in the mix of these five cytokines together with Flt3L and the anti-CD40 mAb. The precursors (20,000) were grown for 4 days in 0.1 ml medium in 96-well, flat-bottomed culture trays. The cultured cells were harvested, pooled, dissociated by EDTA treatment and then stained with mAb, generally in two fluorescent colours. Dead or damaged cells, approximately 15% of the total, were excluded from analysis by propidium iodide staining and forward light scatter characteristics. The surface phenotype of the dendritic cells produced was similar under these two conditions. It was also largely unchanged when day 6 cultures were compared to day 4 cultures.

Of the characteristic T cell markers, the cells from these cultures lacked CD4, CD8α, CD8β, CD3, and CD25. This implied they had lost the low level of CD4 characteristic of the precursors, but had not progressed to the next downstream CD25+ precursor, nor had they formed mature T cells. Surprisingly, they had not gained CD8α, a marker characteristic of most thymic dendritic cells. The cells were Thy 1 positive, showing a wide range of Thy 1 expression; however, since many types of cultured cells express Thy 1, this marker is not useful for defining T lineage cells in culture.

Of the B lymphocyte lineage markers, the cells lacked B220, and also lacked BP- 1, an early B-cell marker expressed on thymic dendritic cells. However, BP-1 is known to be induced on dendritic cells by the thymic environment, and the low CD4 precursors produce BP-1-DC if allowed to develop in the spleen.

Of the macrophage/granulocyte markers, the cells were negative for Gr-1. They stained at levels varying from negative to moderately positive for CD11b (Mac-1) and negative to moderately positive for F4/80. The very strong staining characteristic of macrophages was not seen with either marker. CD11b is expressed at levels ranging from low to high on different lymphoid tissue dendritic cells (Ismaili *et al.*, 1996; Shortman & Wu, 1996). F4/80 has been observed on cultured dendritic cell precursors, but is not normally expressed at this level on mature tissue dendritic cell (Vremac *et al.*, 1992).

Of the typical dendritic cell markers, the cultured cells expressed very high levels of class I and class II MHC, high levels of CD11c and moderate levels of DEC205. They expressed B7- 1 (CD80) and B7-2 (CD86), characteristics of mature dendritic cells. As do most dendritic cells, they expressed CD40, CD44 and HSA, the heat stable antigen; these markers are also found on other cell types. They also had the high forward and side scatter characteristic of dendritic cells, as expected from their size and appearance in. Overall they resembled mature dendritic cells, the only anomaly being the absence of CD8, a marker characteristic of thymic dendritic cells in the mouse but present on only about half of splenic or lymph node dendritic cells. However, there is no evidence that CD8 has any role in either the function or the development of dendritic cells. It should be noted that the low CD4 precursors lack surface class II MHC, CDllc, DEC205, CD80 and CD86, so differentiation towards expression of these dendritic cells markers had occurred in the cultures.

EXAMPLE 18

T Cell Stimulatory Activity of the Cultured dendritic cells

To determine if the culture system produced functional dendritic cells, the dendritic cells produced from thymic precursors by culture in the presence of IL-1β, TNFα, IL-3, IL-7, SCF, Flt3L and mAb binding CD40 were compared with freshly isolated thymic dendritic cells in their ability to stimulate the proliferation of allogeneic CD4 T cells. The small scale mixed leukocyte cultures used (Suss & Shortman, 1996) involved 20,000 pure $CD4^+$ lymph node T cells as responders, and small numbers of either pure thymic dendritic cells

or cultured dendritic cells as stimulators; no exogenous cytokines were added. Both the dendritic cells cultured with the full seven cytokine mix and the normal thymic dendritic cells were found to be efficient stimulators of mature T cell proliferation; both gave a proliferative response peaking at day 3 to 3.5, and both gave a good dendritic cells dose/response relationship and a very high stimulation index (Fig. 1). The dendritic cells cultured with the full cytokine mix gave a slightly better T cell stimulation than freshly isolated thymic dendritic cells. Dendritic cells cultured in the absence of Flt3L and anti-CD40, although giving good stimulation at low dendritic cells levels, gave a reduced proliferation compared to freshly isolated thymic dendritic cells above 1000 dendritic cells per culture.

EXAMPLE 19

Frequency of Responding and Dendritic Cells-Producing Precursors

The rapid increase in cell counts during the response of the low CD4 precursor preparation to the cytokine mixes suggested that a significant proportion of the cells were responding. However, since it required around 50 precursors to form one dendritic cell cluster at day 4, it may have been that only 2% of the precursor population was committed to dendritic cell development. The argument against this was that the incidence of cells with dendritic cell morphology was very much higher than 2% early in the culture, and that the clusters represented aggregates, rather than colonies derived from a single precursor. To determine the actual incidence of responding cells, a series of experiments was conducted where the low CD4 precursors were set up in Terasaki tray cultures at the 1 cell per well level in medium containing the optimum mix of "cytokines" (IL-1β, TNFα, IL-3, IL-7, SCF, Flt3L and anti-CD40 antibody). After 2 hr the cultures were examined under inverted phase microscopy, and cultures with a single precursor in the well were selected for further day by day observation. In one such experiment the fate of 27 single precursor cells was followed over 3 days. By day 1, 9 of the starting cells had died, but all but one of the remaining viable cells showed a clear dendritic cell morphology; of these 70% survivors with dendritic cell morphology, 20% had already undergone one cell division. By day 3, one further culture had terminated in cell death, but all the remainder

had divided to produce between 2 and 10 progeny; 90% of the viable progeny cells in these clones were of dendritic cell morphology. In some cases the progeny cells stayed associated as a mini-cluster, but in many cultures they moved apart. Two subsequent experiments confirmed these observations. Thus the majority (at least 70%) of the cells in the low CD4 precursor preparation could be considered as potential dendritic cell precursors. It was of interest that differentiation of these low CD4 precursors to a dendritic cell morphology was rapid and usually preceded cell division.

EXAMPLE 20

Growth and Dendritic Cell Development from Successive T Precursor Populations

When the earliest adult mouse thymus T precursor population, the low CD4 precursor ($CD4^{lo}8^-3^-25^-44^+$c-kit$^+$), was cultured in the seven cytokine mix, extensive cell growth and dendritic cell development occurred as described previously (Saunders *et al.*, 1996). Some initial cell death occurred, shown previously by single cell culture to represent 25-30% of the total cells. After one day cell division and expansion commenced; at this stage cells had already began to display cytoplasmic extensions and a general dendritic cell morphology. Cell expansion was maximal at day 4 of culture, representing 4 to 5-times the original input, or around 7-times the initial surviving cell number. At this time the cells were in the form of tight clusters, difficult to completely dissociate, so these counts may have been an underestimate. Almost all (>95%) of the cells at this time had dendritic morphology under phase contrast microscopy. The numbers of viable cells declined after day 4, but a further 5-fold expansion could be achieved by splitting the cultures at day 3-4 and reculturing in fresh medium with fresh cytokines. However, after a second reculture, dendritic morphology was lost, cell expansion was reduced and extensive cell death occurred.

When the next downstream T precursor population, the pre-T cells ($CD4^-8^-3^-25^+44^+$c-kit$^+$) (Shortman & Wu, 1996; Godfrey & Zlotnik, 1993), was cultured under identical conditions, there was a more extensive loss of cell viability over the first day,

amounting to around 70% of the initial cell input. After this initial cell drop, cell division and expansion commenced at day 1 and cells with dendritic morphology appeared in the cultures. The cell expansion reached a peak at day 4, where the cells were in the form of tight clusters and >96% of the cells had dendritic morphology. Although the overall yield of cells compared to the total initial input was much lower than with the low CD4 precursors, there was a similar level of expansion and dendritic cells development from the viable cells surviving at day 1. The main difference between the cultures of the two precursor populations was therefore the level of initial cell death.

When the third successive mouse thymus precursor population, the pre-T cell ($CD4^-8^-3^-25^+44^-c\text{-}kit^-$) (Shortman & Wu, 1996; Godfrey & Zlotnik, 1993), was cultured, very different results were obtained. The cells showed an extensive loss of viability over the first day and this loss continued thereafter. No cell expansion was obtained and there were few, if any, dividing cells in the cultures. Of the viable cells that survived, very few exhibited dendritic morphology.

When the final precursor population, the "late double negatives" ($CD4^-8^-3^-25^-44^-c\text{-}kit^-$) (Shortman & Wu, 1996; Godfrey & Zlotnik, 1993), was cultured, the results differed slightly from that obtained with the previous precursor population. Again there was extensive initial cell death. However, a proportion of the cells did proliferate and some expansion of cell numbers was evident by day 4. This expansion was more marked in more concentrated "bulk" cultures than in the 250 cell Terasaki cultures, suggesting it was in part driven by endogenously produced cytokines. However, despite this limited cell expansion, very few of the cells displayed dendritic morphology.

EXAMPLE 21

Surface Markers on Cultured Cells Derived from Successive T Precursors

The cell surface phenotype of the cultured cells was determined by harvesting at day 4, immunofluorescent staining and flow cytometric analysis. The surface phenotype of the cells derived from the low CD4 precursors and the pro-T cells was similar, and confirmed that in both cases the cells produced were dendritic cells. They expressed high levels of class II MHC, of CD11c and of CD44. They were positive for DEC-205, although the level of expression was lower than for normal thymic dendritic cells. They expressed moderate levels of CD80 (B7/1) and CD86 (B7/2), suggesting they were mature dendritic cells. They lacked T cell or B cell markers and were relatively low or negative for myeloid markers. CD11b was expressed, but this is present at moderate levels on certain dendritic cells and is upregulated on all murine lymphoid-organ derived dendritic cells on culture.

In contrast, the cells surviving at day 4 in cultures of the pre-T cells or the late double negatives were class II MHC low and CD11c low, confirming the morphological assessment that they were not dendritic cells.

The surface phenotype analysis gave some clues to the nature of the few cells produced by proliferation in the late double negative precursor cultures. They expressed low levels of CD4 and a proportion expressed both CD8α and CD8β, many expressed CD3 and a small proportion expressed TCRγδ. This suggested many of the proliferating cells were of the T lineage, and some were γδ T cells. In previous results from this laboratory γδ T cells have been produced in culture from late double negatives under the influence of IL-2 (Petrie *et al.*, 1992). Thus the limited expansion and differentiation obtained from this population may have been driven by endogenously produced IL-2, in conjunction with some of the cytokines added. However, the cytokine mix employed was clearly not optimal for T-cell differentiation from these late precursors, given the limited proliferation and differentiation achieved.

EXAMPLE 22

Yield of dendritic cells from Different T Precursor Populations: *in vivo* and *in vitro* Comparison

The yield of dendritic cells from the successive T precursors around the peak of the response, either at 4 days of culture or in recipient spleens or thymuses 14 days after intravenous transfer, is compared in Table 5. The cell culture and the cell transfer results agree in the general conclusion that dendritic cells can be produced from both the low CD4 precursors and the pro-T cells, but not from the pre-T cells or the late double negatives. There is some disagreement regarding the relative efficiency of dendritic cells production by the two earlier precursor populations, the culture results indicating a marked reduction as precursors develop to the $CD4^{-}8^{-}25^{+}44^{+}c\text{-}kit^{+}$ stage whereas the transfer studies suggest a near equivalent efficiency in the thymus but a substantial drop in efficiency in the spleen. This might be the result of the extensive death of the pro-T cells in culture, which may not occur *in vivo*. Alternatively, differences in the efficiency of seeding different organs by the two precursor populations could explain the discrepancy. The differences in the peak havest time for dendritic cell development (4 days in culture, 7-14 days in the thymus or spleen) is a third variable.

EXAMPLE 23

The Expression of CD8α and BP-1 on Thymic dendritic cells Generated *in vitro* or *in vivo*

The lack of any expression of BP-1, and the very low to negative expression of CD8α on the dendritic cells generated in culture from the thymic precursors presented a paradox. These two surface proteins, and the mRNAs for their formation, are expressed by normal thymic dendritic cells (Wu *et al.*, 1995; Vremac *et al.*, 1992). We have shown previously (Wu *et al.*, 1996) that BP-1 is expressed on the dendritic cells progency of the low CD4 precursors if they seed in the thymus, but not if they

seed in the spleen, indicating that some factor in the the thymus induces expression of this marker; perhaps it is not surprising that this factor is missing in culture. However, CD8α is present on the dendritic cells progeny of both early precursors, regardless of whether they develop in the thymus (Wu *et al.*, 1995; 1996) or in the spleen (Fig. 2). In contrast, the dendritic cells which develop from these thymic T precursors in culture resemble conventional myeloid-derived dendritic cells by most surface markers and lack CD8α. In view of the high efficiency of dendritic cell production in culture it seems unlikely that different subpopulations of the precursors function *in vitro* than function *in vivo*. It is more likely that a factor inducing CD8α expression is present in both spleen and thymus, but missing in our cultures. In support of this, the dendritic cells progeny found in the lymph nodes after intravenous transfer of these precursors includes a proportion of $CD8\alpha^-$ and $CD8\alpha^{lo}$ dendritic cells, as well as the $CD8\alpha^{hi}$ dendritic cells. Attempts to induce CD8α expression on the cultured dendritic cells by the addition of other cytokines (including TGFβ) have so far proved unsuccessful. However, there is no evidence that CD8α is essential for either the development or the function of dendritic cells.

EXAMPLE 24

Dendritic cell production in culture: different precursors, different cytokines

Precursor cells, either the mouse thymic low CD4 precursor or mouse bone marrow cells depleted of all cells bearing lineage markers of mature blood cells (Lin^- Bone Marrow, an enriched source of precursor cells) (Wu *et al.*, 1991; Wu *et al.*, 1996) were cultured at a level of 250 cells per well in Terasaki culture trays. The cultures were stimulated either with GM-CSF and TNFα (the cytokine mix usually used to grow myeloid-related dendritic cells) or with the mix of cytokines found to be optimum for production of dendritic cells from the thymic lymphoid-precursor populations [TNFα, IL-1β, IL-3, IL-7, SCF, Flt-3L, anti-CD40 antibody]. At day 4, the total number of cells in the cultures showing dendritic morphology was counted under phase contrast microscopy. Results are means ±SEM of 20 cultures.

GM-CSF and TNFα gave significant dendritic cell production from bone marrow precursors, but not from the thymus precursors (Table 6). The seven cytokine mix lacking GM-CSF grew out dendritic cells from both sources. The results suggest the thymic precursors were of one type only (lymphoid-related precursors) whereas the bone marrow precursors were of two types (lymphoid-related and myeloid-related precursors). The results also indicate that dendritic cell precurors responsive to the seven cytokine mix are present in tissues other than the thymus.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

TABLE 1

The effects of antibody against GM-CSF on the generation of dendritic cells clusters by cultured thymic low CD4 precursors

Cytokine mix	Anti-GM-CSF	Clusters per well
TNFα+IL-1β+IL-7+SCF+IL-3	-	20±2
	+	18±2
TNFα+IL-1β+IL-7+SCF	-	22±2
	+	19±1

Purified thymic low CD4 precursors were cultured at 3000 cells per well in 0.01 ml medium for 4 days with the cytokines listed. Results are the means ± SEM of pooled data from 3 experiments, each with 5 cultures per assay.

TABLE 2

The effects of various cytokine combinations on the generation of dendritic cells clusters in low density cultures of thymic precursors

Cytokines	Clusters per culture
No cytokines	0±0
IL-1β	0±0
TFNα	0±0
TNFα+IL-1β	0±0
IL-1β+TNFα+SCF	0±0
IL-1β+TNFα+IL-3	0.4±0.6
IL-1β+TNFα+IL-7	0.1±0.2
IL-1β+TNFα+IL-7+SCF	0.1±0.2
IL-1β+TNFα+IL-7+IL-3	0.2±0.4
IL-1β+TNFα+SCF+IL-3	0.4±0.6
IL-7+SCF+IL-3	0.1±0.1
IL-1β+TNFα+IL-7+SCF+IL-3	2.0±0.9

Cultures of 250low CD4 precursors were set up in 0.01 ml medium in Terasaki tray wells. The incidence of dendritic cells clusters was determined after 4 days of culture. Results are the means ± SEM of data pooled from 2 experiments, each with 20 cultures per condition.

TABLE 3

The growth and development of dendritic cells clusters from thymic low CD4 precursors from GM-CSF "null" mice

	Control C57BL/6 precursors	GM-CSF null precursors
Cells per culture	1002±109	721±69
Clusters per culture	5.7±1.6	4.7±1.6

Purified low CD4 precursors, 250 per 0.01 ml medium in Terasaki culture wells, were cultured for 4 days in the presence of TNFα, IL-1β, IL-3, IL-7, SCF, Flt3L and FGK45.5 mAb against CD40. Results are the means ± SEM of pooled data from 2 experiments, each with 20 cultures per determination.

TABLE 4

The influence of GM-CSF and IL-3 on dendritic cells development from low CD4 thymic precursors in low density cultures

Cytokines	Cells per culture day 4	Clusters per culture day 4
IL-1β, TNFα, IL-7, SCF, Flt-3L, anti-CD40 alone	4,930	1.8±0.4
IL-1β, TNFα, IL-7, SCF, Flt-3L, anti-CD40 plus IL-3	10,130	4.3±0.4
IL-1β, TNFα, IL-7, SCF, Flt-3L, anti-CD40 plus GM-CSF	6,570	3.7±0.4
IL-1β, TNFα, IL-7, SCF, Flt-3L, anti-CD40 plus IL-3 and GM-CSF	8,360	4.6±0.5

Purified low CD4 thymic precursors were cultured at 250 cells per well in 0.01 ml medium for 4 days with the cytokines listed. Results are the pooled data from 2 experiments, each with 20 cultures per condition. Cluster counts are the means ± SEM of individual direct culture counts. Cell counts were performed on the pool of the 20 cultures in each experiment after harvest and the results are the mean of the 2 experiments.

TABLE 5

Precursor population	Culture DC per 10^6 precursors day 4	Transfer i.v. Thymus DC per 10^6 precursors day 14	Transfer i.v. Spleen DC per 10^6 precursors day 14
$CD4^{lo}$	$6.1 \pm 2.3 \times 10^6$	$0.4 \pm 0.1 \times 10^6$	$0.4 \pm 0.7 \times 10^6$
$CD25^+$ c-kit^+	$1.8 \pm 0.8 \times 10^6$	$0.4 \pm 0.2 \times 10^6$	$0.4 \pm 0.1 \times 10^6$

TABLE 6

Dendritic Cell Production

	TNFα, IL1, IL3, IL7 SCF, Flt3L, anti-CD40	GM-CSF + TNFα
Low CD4 Thymus precursor	906 ± 57	2 ± 3
Lin-Bone marrow	864 ± 37	641 ± 69

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CLAIMS:

1. A method of developing dendritic cells from precursor cells, said method comprising culturing said precursor cells in the presence of an effective amount of a mixture of at least three cytokines or functional derivatives thereof wherein at least two cytokines are TNFα and IL-1 or functional derivatives thereof.

2. A method according to claim 1 wherein at least three cytokines are TNFα, IL-1 and IL-3 or functional derivatives thereof.

3. A method according to claim 1 or 2 wherein said IL-1 is IL-1β or a functional derivative thereof.

4. A method according to claim 3 wherein said mixture comprises TNFα, IL-1β and IL-3 and an effective number of cytokines selected from IL-7, SCF, Flt3L, CD40L and an anti-CD40 antibody, or functional derivatives thereof.

5. A method according to claim 4 wherein said mixture comprises TNFα, IL-1β, IL-3, IL-7, SCF and Flt3L or functional derivatives thereof.

6. A method according to claim 4 wherein said mixture comprises TNFα, IL-1β, IL-3, IL-7 and SCF or functional derivatives thereof.

7. A method according to claim 4 wherein said mixture comprises TNFα, IL-1β, IL-3, IL-7, SCF, Flt3L and CD40L or functional derivatives thereof.

8. A method according to claim 4 wherein said mixture comprises TNF-α, IL-1β, IL-3, IL-7, SCF, Flt3L and an anti-CD40 antibody or functional derivatives thereof.

9. A method according to claim 4 wherein said mixture comprises TNFα, IL-1β, IL-3, IL-7, SCF, Flt3L and FGK45.5 or functional derivatives thereof.

10. A method according to claim 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 wherein said precursor cells are lymphoid precursor cells.

11. A method according to claim 10 wherein said lymphoid precursor cells are T cell precursor cells.

12. A method according to claim 11 wherein said T cell precursor cells are CD4 low precursor cells.

13. A method according to claim 11 wherein said T cell precursor cells are pro-T cells.

14. A mixture for use in developing dendritic cells from precursor cells said mixture comprising at least three cytokines or functional derivatives thereof wherein at least two

cytokines are TNFα and IL-1.

15. The mixture according to claim 14 wherein at least three cytokines are TNFα, IL-1 and IL-3 or functional derivatives thereof.

16. The mixture according to claim 14 or 15 wherein IL-1 is IL-1β or a functional derivative thereof.

17. The mixture according to claim 16 wherein said mixture comprises TNFα, IL-1β and IL-3 and an effective number of cytokines selected from IL-7, SCF, Flt3L, CD40L and an anti-CD40 antibody or functional derivatives thereof.

18. The mixture according to claim 17 wherein said mixture comprises TNFα, IL-1β, IL-3, IL-7, SCF and Flt3L or functional derivatives thereof.

19. The mixture according to claim 17 wherein said mixture comprises TNFα, IL-1β, IL-3, IL-7 and SCF or functional derivatives thereof.

20. The mixture according to claim 17 wherein said mixture comprises TNFα, IL-1β, IL-3, IL-7, SCF, Flt3L and CD40L or functional derivatives thereof.

21. The mixture according to claim 17 wherein said mixture comprises TNF-α, IL-1β, IL-3, IL-7, SCF, Flt3L and an anti-CD40 antibody or functional derivatives

thereof.

22. The mixture according to claim 18 wherein said mixture comprises TNFα, IL-1β, IL-3, IL-7, SCF, Flt3L and FGK45.5 or functional derivatives thereof.

23. A method of treating a subject said method comprising administering to said subjet an effective amount of a mixture of at least three cytokines or functional derivatives thereof for a time and under conditions wherein said cytokines induce dendritic cell development from precursor cells.

24. A method according to claim 23 wherein at least two cytokines are TNFα and IL-1 or functional derivatives thereof.

25. A method according to claim 23 wherein at least three cytokines are TNFα, IL-1 and IL-3 or functional derivatives thereof.

26. A method according to claims 24 or 25 wherein IL-1 is IL-1β or a functional derivative thereof.

27. Use of a mixture of at least three cytokines in the manufacture of a medicament for the induction of dendritic cell development from precursor cells in a subject wherein at least two cytokines are TNFα and IL-1 or functional derivatives thereof.

28. Use of a mixture of at least three cytokines in the manufacture of a medicament for the induction of dendritic cell development from precursor cells in a subject wherein at least three cytokines are TNFα, IL-1 and IL-3 or functional derivatives thereof.

29. Use of a mixture according to claims 27 or 28 wherein IL-1 is IL-1β or a functional derivative thereof.

30. A pharmaceutical composition comprising a mixture of at least three cytokines or functional derivatives thereof wherein said cytokines induce dendritic cell development from precursor cells together with one or more pharmaceutically acceptable carriers and/or diluents.

31. A pharmaceutical composition according to claim 30 wherein at least two cytokines are TNFα and IL-1 or functional derivatives thereof.

32. A pharmaceutical composition according to claim 30 wherein at least three cytokines are TNFα, IL-1 and IL-3 or functional derivatives thereof.

33. A pharmaceutical composition according to claims 31 or 32 wherein said IL-1 is IL-1β or a functional derivative thereof.

34. Use of dendritic cells in the manufacture of a medicament for the treatment of a subject.

35. A method of treating a subject said method comprising administering to said subject an effective number of dendritic cells wherein said dendritic cells are produced by the method of the present invention.

36. The dendritic cells produced by the method of claim 1.

37. An agent for use in developing dendritic cells from precursor cells comprising a mixture of at least three cytokines or functional derivatives thereof wherein at least two cytokines are TNF-α and IL-1 or functional derivatives thereof.

38. An agent according to claim 37 wherein said IL-1 is IL-1β or a functional derivative thereof.

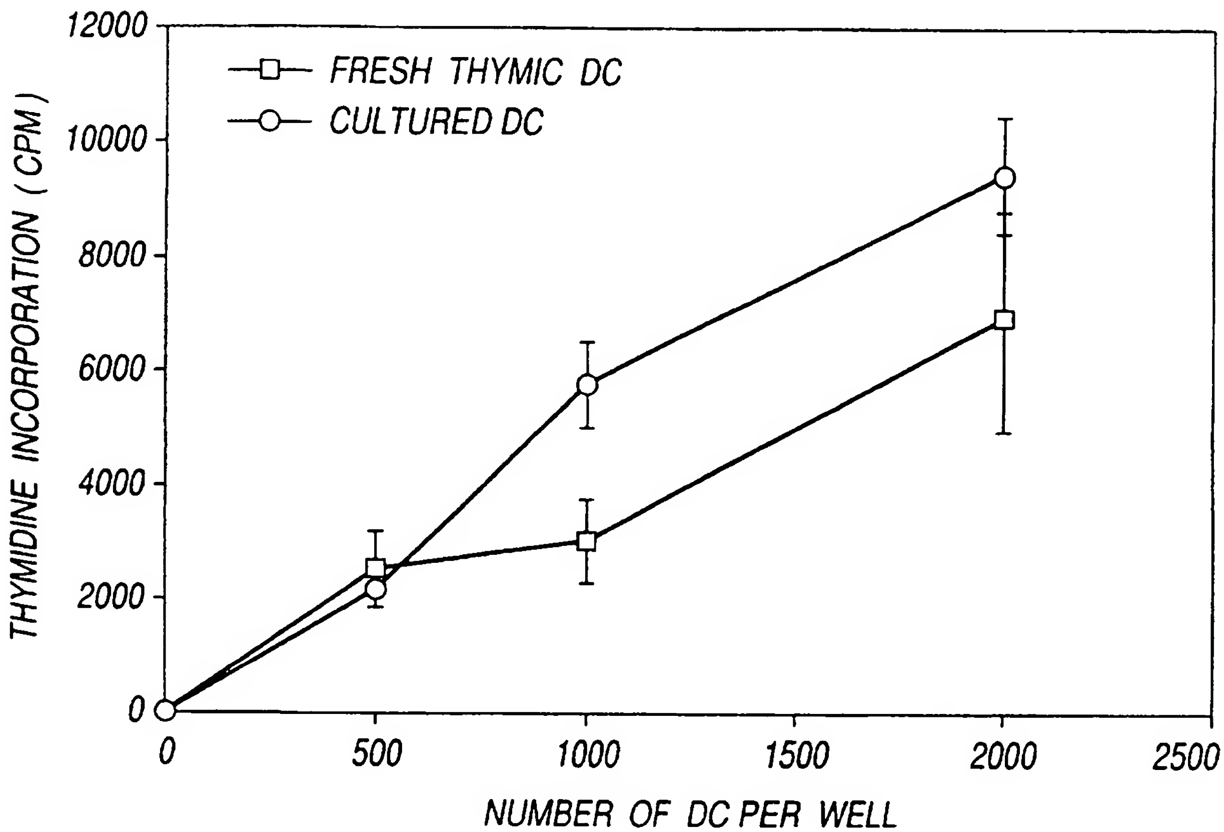
12000
10000
8000
6000
4000
2000
0
THYMIDINE INCORPORATION (CPM)
FRESH THYMIC DC
CULTURED DC
0
500
1000
1500
2000
2500
NUMBER OF DC PER WELL

Fig.1

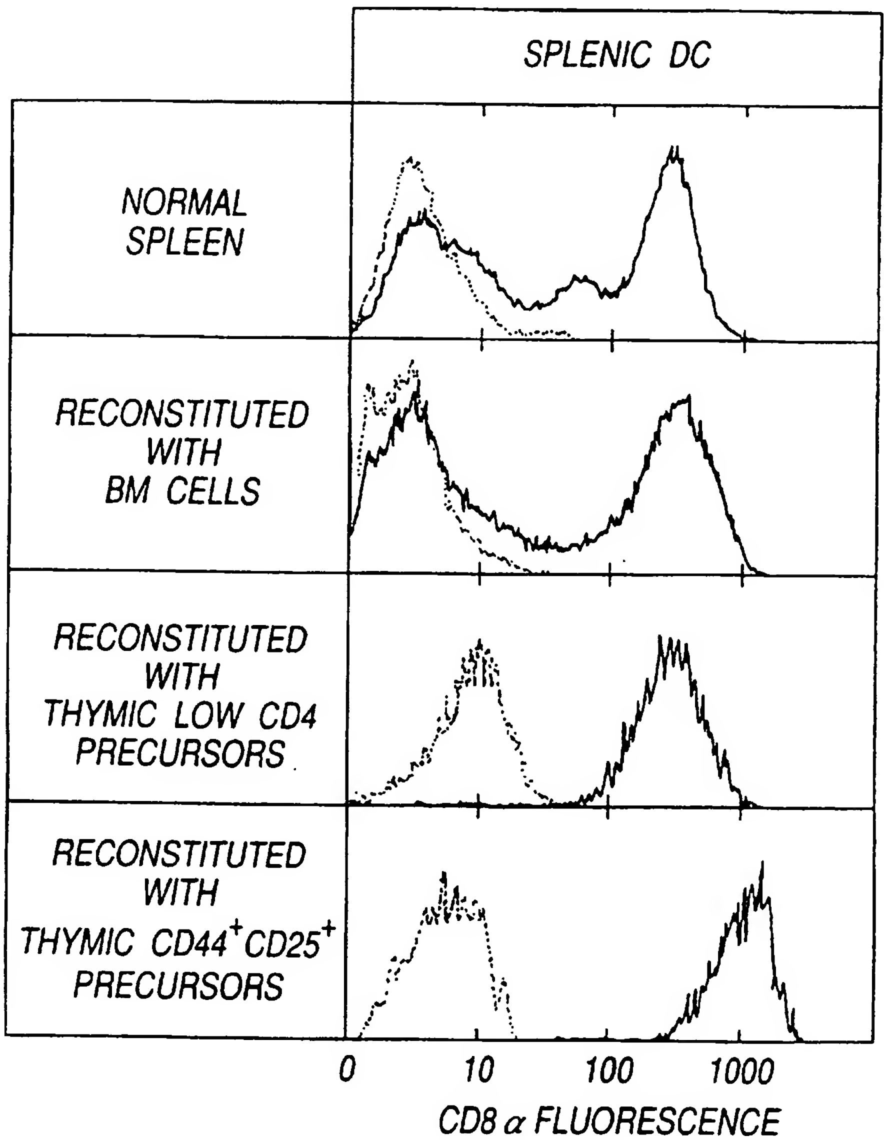
SPLENIC DC
NORMAL SPLEEN
RECONSTITUTED WITH BM CELLS
RECONSTITUTED WITH THYMIC LOW CD4 PRECURSORS
RECONSTITUTED WITH THYMIC CD44+CD25+ PRECURSORS
0
10
100
1000
CD8 α FLUORESCENCE

Fig.2

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00647

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl^{6}: C07K 14/525, 14/545, A61K 38/19, 38/20, C12N 5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT (Interleukin 1, Tumour necrosis factor)
Medline (Dendritic cells, Interleukin 1, Tumour necrosis factor)
CA (Lymphokines and Cytokines, Interleukin 1, Tumour necrosis factor, Dendritic cells)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF IMMUNOLOGY, Volume 149, Number 8, 1992, C. D. L. Reid et al., 'Interactions of Tumour Necrosis Factor with Granulocyte-Macrophage Colony- Stimulating Factor and Other Cytokines in the Regulation of Dendritic Cell Growth In Vitro from Early Bipotent CD34^{+} Progenitors in Human Bone Marrow', pages 2681-2688 see in particular abstract	1-22, 36-38
X	WO 92/07578 (GENENTECH, INC.) 14 May 1992 see in particular claims	14, 16, 23-27, 29-31, 33, 37, 38

☐ Further documents are listed in the continuation of Box C

☐ See patent family annex

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 November 1997	19 NOV 1997

Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	L. F. Mc CAFFERY Telephone No.: (02) 6283 2573

INTERNATIONAL SEARCH REPORT
Information on patent family members

International Application No.
PCT/AU 97/00647

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Patent Document Cited in Search Report	Patent Family Member (To put a line under the citations tab to the first point on the next row and press F8)					
WO 92/07578	AT	133073	AU	89412/91	CA	2092718
	DE	69116565	EP	554381	ES	2084190
	JP	6503320				

END OF ANNEX

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